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Genetics of *Heliothis* and *Helicoverpa* Resistance to Chemical Insecticides and to *Bacillus thuringiensis**

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Abstract: Genetic linkage maps of *Heliothis virescens* and *Helicoverpa armigera* are being used to identify and characterize resistance-conferring genes. The insensitive acetylcholinesterase conferring resistance to organophosphorus insecticides and the insensitive sodium channel conferring resistance to pyrethroids have both been mapped in *H. virescens*. The linkage mapping approach permits a genetic dissection of resistance, even when the mode of action and lethal target are not precisely known, such as for the insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt). We have identified and mapped a major Bt-resistance locus in a strain of *H. virescens* exhibiting up to 10 000-fold resistance to Cry1Ac toxin and are currently developing a linkage map for *H. armigera* with a set of 'anchor' loci to facilitate comparison with *H. virescens*. Both species are currently experiencing their first significant selective pressure in the field by transgenic cotton expressing Cry1Ac, and timely identification of resistance mechanisms and their underlying genetic basis will be essential in successfully managing the Bt resistance that will eventually appear.

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1 INTRODUCTION

Heliothis and *Helicoverpa* are two genera of heliothine, noctuid moths that contain some of the most destructive agricultural pests in the world. These include the tobacco budworm *Heliothis virescens* F. and the corn earworm *Helicoverpa zea* Boddie in the Americas, the cotton bollworm *Helicoverpa armigera* (Hübner) worldwide exclusive of the Americas, and the bollworm *Helicoverpa punctigera* (Wall.) in Australia. Crops attacked by this species complex include cotton, tobacco, maize, chickpeas, pigeonpeas and tomatoes. All four species were formerly included in the genus *Heliothis;* however taxonomic revision has led to the recognition of a new genus *Helicoverpa.*[1] Including these notorious pests, the genus *Heliothis* consists of 80–90 species and *Helicoverpa* 20–25 species worldwide.

One particularly troublesome aspect of *H. virescens* and *H. armigera* is their ability to develop resistance to insecticides. Resistance to DDT, organophosphorus insecticides and synthetic pyrethroids has bedeviled attempts at chemical pest control and, in some cases, has forced abandonment of cropping due to the inability to control the pest. For students of insecticide resistance, these species provide an abundance of resistance mechanisms to explore, and a challenge to apply this basic knowledge to design effective and practical strategies for resistance management and prevention.

Although much useful general information can be gained by studying insecticide resistance mechanisms in model systems such as *Drosophila melanogaster* Meig.,[2] the specific information required to understand and manage insecticide resistance in crop pests must be based on genetical studies of the pests themselves. This rationale motivates our current research programme in the heliothine pest species, which focuses on linkage mapping of resistance mechanisms. Over the past several years we have developed a linkage map for *H. virescens* and have applied it to the study of well-known mechanisms of resistance to organophosphorus and pyrethroid insecticides. More recently, we are applying the map to the genetic analysis of the less-well-understood resistance to insecticidal toxins from the bacterium *Bacillus thuringiensis* Berliner (Bt). Moreover, we have begun to extend the mapping approach to *H. armigera*, in which problems of resistance are no less pressing. In both species, delaying and managing resistance to Bt toxins expressed by transgenic crops could be greatly hindered by our incomplete knowledge of the mode of action and resistance mechanisms. The linkage mapping approach offers unique advantages in identifying and cloning Bt resistance genes and in applying information derived from one species to studying resistance mechanisms in others.

2 STATUS OF THE LINKAGE MAP IN *HELIOTHIS VIRESCENS*

H. virescens has a haploid chromosome number of $n = 31$[3] and a genome size of 403×10^6 base pairs.[4] Currently, approximately 350 marker loci have been assigned to one of the 31 linkage groups corresponding to the chromosomes (Heckel, D. G. and Gahan, L. J., unpublished). These marker loci include 18 allozyme loci disclosed by starch gel electrophoresis and enzyme-specific staining, 10 RFLPs defined by anonymous single-copy genomic clones, 9 RFLPs defined by known genes, 110 RAPD markers[5] and 206 AFLP markers.[6] Initial identification of all 31 linkage groups was greatly facilitated by an interspecific cross between *H. virescens* and *Heliothis subflexa* Guenée,[7] and utilization of the fact that in *Heliothis* (as in most or all Lepidoptera) crossing-over is limited to males.[8,9] Fine-scale mapping of markers within linkage groups is still in progress. Because the chromosomes of *Heliothis* (as of most Lepidoptera) are highly condensed and indistinguishable by current methods, the actual correspondence between linkage groups and chromosomes is not known. Since each chromosome consists of only about 3% of the entire genome, linkage group assignment allows a moderately high level of resolution in mapping.

So far the most frequent use of the linkage map has been to determine the number and identity of linkage groups contributing to resistance to a particular insecticide in a particular strain. Linkage groups are identified using specific marker loci previously localized to them. Resistance can be measured directly by bioassay or indirectly by measuring some property of a resistance mechanism. Quantitative, rather than qualitative, measures of resistance can provide the extra information necessary to distinguish between resistance genes of major versus minor effect. Depending on the resolution of the mapping study, it may be possible to further discern the number of resistance genes within a linkage group. Even without this higher resolution, the high chromosome number makes linkage-group assignment of resistance mechanisms a powerful technique for characterizing them in different strains, even when multiple mechanisms are present simultaneously.

3 INSENSITIVE ACETYLCHOLINESTERASE AND RESISTANCE TO ORGANOPHOSPHATES IN *HELIOTHIS VIRESCENS*

By the late 1960s, a combination of DDT resistance and environmental concerns had forced a switch from DDT to organophosphorus (OP) insecticides by American cotton growers to control *H. virescens.*[10] Extensive use of OPs, particularly parathion-methyl, selected for higher resistance levels during the 1970s, and at the end of that decade OPs were generally abandoned in favor

of the synthetic pyrethroids. Studies of OP resistance mechanisms found evidence for reduced penetration, reduced activation, increased detoxification and decreased insensitivity of acetylcholinesterase (AChE), the primary target of OP insecticides.[11–14]

By reacting with the active site of the enzyme, OP insecticides inhibit the hydrolysis of the neurotransmitter acetylcholine by AChE.[15] As a response to insecticide selection pressure, a number of insect species have developed forms of AChE that are much less sensitive to inhibition.[16] Insensitive AChE is the primary mechanism of parathion-methyl resistance in the Woodrow strain of *H. virescens* from South Carolina.[14] Utilizing a colorimetric assay of AChE that permitted simultaneous testing of sensitivity to multiple inhibitors in head homogenates of single individuals, Brown and Bryson[14] showed that a single locus (*AceIn*, controlling acetylcholinesterase inhibition) with two alleles sufficed to account for the phenotypes observed. The single-locus nature of this trait was confirmed when *AceIn* was shown to be genetically linked to *IDH-2*, the locus encoding the cathodal isozyme of isocitrate dehydrogenase.[17,18] This result permitted assignment of *AceIn* to Linkage Group 2, the first linkage assignment of an insecticide resistance gene in any lepidopteran species.

Subsequent isolation of the resistant and susceptible alleles of *AceIn* in two strains with a similar, randomized genetic background showed that AChE insensitivity accounted for a resistance factor of 16-fold, compared to the 130-fold resistance level of the Woodrow strain.[19] This indicated that other resistance mechanisms besides *AceIn* had an effect on parathion-methyl resistance. Population surveys have shown that the frequency of the resistant allele at *AceIn* has declined from about 85% to approximately 14% in the years following cessation of parathion-methyl use in the southeastern United States.[20]

The linkage assignment of *AceIn* to Linkage Group 2 permits a novel prediction on the nature of insensitive AChE as a resistance mechanism in certain tortricid moths.[18] It appears that the homologue of the autosomal Linkage Group 2 of *H. virescens* has become translocated to the sex-chromosome in some species in the family Tortricidae. This leads to the prediction that if the mechanism of OP or carbamate resistance in these species is due to an insensitive target AChE, the resistance should be sex-linked. This prediction is novel because cases of sex-linked insecticide resistance are very rare (although a few cases are known[21–24]).

4 INSENSITIVE SODIUM CHANNEL AND RESISTANCE TO PYRETHROIDS IN *HELIOTHIS VIRESCENS*

In the 1980s, synthetic pyrethroids replaced parathion-methyl for control of *H. virescens* in US cotton. In anticipation of resistance eventually developing in field populations, efforts were made to select for resistant strains in the laboratory so that mechanisms could be studied. One of the earliest such strains was the RR strain, produced by selection and single-pair matings from a collection originating in 1982.[25,26] Resistance in this strain was reduced, but not completely overcome, by propynyl aryl ether inhibitors of cytochromes P450,[27] and there was also evidence of nerve insensitivity as a resistance mechanism.[28] Genetic analysis of RR produced a complex picture, with evidence of a major locus and one or more minor loci responsible for the resistance to permethrin.[25] A preliminary linkage analysis found that Linkage Groups 10 and 3 both made a detectable contribution to resistance (Heckel, D. G., unpublished).

The voltage-gated sodium channel appears to be the primary target of pyrethroids.[29] Following the discovery of two distinct sodium channel genes in *Drosophila*, a portion of the homologue of the *para*-type was cloned from *H. virescens*. Linkage analysis showed that Linkage Group 10 containing this gene accounted for about 50% of the total resistance in the RR strain.[26] Similar linkage results in housefly[30] and cockroach[31] have confirmed the role of an altered *para*-homologous sodium channel in conferring pyrethroid resistance.

The altered sodium channel presumably confers the phenotype of nerve insensitivity, in which resistant strains retain normal firing activity in preparations of the nervous system exposed to pyrethroid concentrations that greatly disrupt the activity in susceptible strains. Nerve insensitivity has been demonstrated in other pyrethroid-resistant strains of *H. virescens* as well.[32–34]

Sequencing the *para*-homologous sodium channel gene from pyrethroid-resistant and susceptible strains of a number of species has indicated which amino acid substitutions may be involved in conferring resistance. Williamson *et al.*[35] found a leucine to phenylalanine substitution in the IIS6 transmembrane region at position 1014 consistently associated with the *kdr* type of pyrethroid resistance in housefly, with all *super-kdr* strains examined having, in addition, a methionine to threonine substitution in the IIS4-S5 loop at position 918. Miyazaki *et al.*[36] observed the same leucine to phenylalanine substitution in a pyrethroid-resistant strain of cockroach, but not in all individuals of a pyrethroid-resistant strain of housefly. Williamson *et al.*[37] have also found the same leucine to phenylalanine substitution in pyrethroid-resistant strains of *Plutella xylostella* (L.) and *Myzus persicae* (Sulz). In *H. virescens*, Park and Taylor[38] found a leucine to histidine substitution at the same position in some, but not all, of the pyrethroid-resistant individuals examined; notably the RR sequence showed no substitution at this position. McCaffery *et al.*[39] and Tan *et al.*[40] reported substitutions at the linker between domains III and IV, but none

at the leucine at position 1014, in another nerve-insensitive pyrethroid-resistant strain of *H. virescens*. Some of these studies found other substitutions in resistant strains as well; thus the question of which mutations cause resistance does not appear to have a simple answer at this stage.

Taylor *et al.*[41,42] have taken an innovative approach to obtaining evidence for selection on the sodium channel in field populations of *H. virescens*. Denaturing gradient gel electrophoresis[43] was used to detect genetic variation in a PCR-amplified fragment of the *para*-homologous sodium channel gene. Fifty different alleles were observed, and the spatial and temporal patterns of this genetic variation supported the hypothesis of recent selection (compared with the patterns observed at an unlinked locus not involved in pyrethroid resistance). An advantage of their approach is that it does not require knowledge of the actual resistance-causing mutations; however selection acting on a different, tightly linked gene could potentially confound the interpretation of these population patterns.

Genetic studies of other pyrethroid resistance mechanisms in *H. virescens* have also been initiated. Recent advances in the cloning of cytochrome P450 genes from insects have facilitated the genetic linkage approach of testing the role of these detoxicating enzymes in insecticide resistance.[44–46] The cytochromes P450 comprise a diverse superfamily of genes in eukaryotes. The first member of the CYP9 family was recently cloned from *H. virescens* and mapped to Linkage Group 7.[47] Although this gene has been implicated in resistance in the strain from which it was isolated, Linkage Group 7 does not make a major contribution to pyrethroid resistance in the RR strain. Cloning and mapping of other P450 genes in *H. virescens* is in progress.

5 BT RESISTANCE IN *HELIOTHIS VIRESCENS*

The previous two examples illustrate the role of linkage studies when information is already available on specific genes suspected to be involved in the toxic action of the insecticide. But a key advantage of the linkage mapping approach is that it permits a genetic dissection of resistance even when the mode of action and lethal target are not precisely known. This is especially important in studying the genetic basis of resistance to the insecticidal crystal proteins produced at sporulation by certain strains of the bacterium *B. thuringiensis*.[48]

Although crystal and spore formulations have been used as sprays for many years, primarily on pests of forests, vegetables and stored grains, only two pest species (*P. xylostella* and *Plodia interpunctella* Hübner) have developed significant resistance levels in the field to date.[49] However, the recent commercial release of transgenic cotton, maize and potatoes expressing Bt toxins will greatly increase the selection pressure, and resistance is expected to develop in many more species, just as it has for chemical insecticides. Laboratory-selected resistant strains have been developed for a number of insect species,[49] showing that Bt resistance is attainable. Although transgenic Bt-expressing plants have many advantages over traditional chemical insecticides in pest control, the threat of resistance is probably the single most important factor compromising their sustainable use.[50]

Compared to other heliothines, *H. virescens* is particularly susceptible to the Cry1Ac toxin, the type currently present in commercially available transgenic cotton. Three Bt-resistant strains of *H. virescens* have been produced by selection in the laboratory using Cry1Ac-impregnated artificial larval diet. The strain developed by Monsanto Company attained 20-fold resistance, with an apparently polygenic mode of inheritance.[51–53] At least three distinct linkage groups contribute to the overall resistance levels in this strain (D. G. Heckel, unpublished). The CP73 strain developed by Gould *et al.*[54] exhibited 50-fold resistance to Cry1Ac, and surprisingly showed cross-resistance to the dissimilar Cry2A toxin, to which it had never been exposed. The strain with the highest levels of CrylAc resistance to date is the YHD2 strain of Gould *et al.*,[55] which is up to 10 000-fold resistant to CrylAc.

Linkage analysis of YHD2 revealed the existence of a major locus (*BtR-4*) on Linkage Group 9 responsible for about 80% of the total Cry1Ac resistance levels.[56] Linkage Group 11 made an additional, smaller, contribution to resistance. Resistance conferred by *BtR-4* is almost completely recessive, with heterozygotes being nearly as susceptible to Cry1Ac as susceptible homozygotes.[55] The linkage assignment of *BtR-4* completes the first step in the positional cloning of this gene.

Many potential Bt-resistance mechanisms are possible at this point,[57] although reduced toxin binding has been the most intensively studied. Bt toxins must bind to specific sites in the larval midgut before creating the pores that lead to lysis of epithelial cells and death. In *P. interpunctella*[58] and *P. xylostella*,[59,60] Cry1Ac resistance is clearly correlated with a loss of the ability of vesicles prepared from the epithelial brush border membrane to bind radiolabeled CrylAc toxin. The situation is much less clear-cut in the YHD2 strain of *H. virescens*.[61] Membrane vesicles from YHD2 bind Cry1Ac and Cry1Ab toxins just as effectively as vesicles from susceptible insects. However, YHD2 shows a marked reduction in Cry1Aa binding. Excess unlabeled Cry1Ac can displace some Cry1Aa from its binding sites. One hypothesis to account for these results is that most Cry1Ac-binding sites in *H. virescens* midgut epithelia do not permit the toxin to form a pore, but the minority that do also bind Cry1Aa. Mutation or deletion of this sub-population of lethal binding sites would cause an observable decline in Cry1Aa binding, but not overall

Cry1Ac binding, and would suffice to protect the cell from the lethal pore-forming action of Cry1Ac.[61] Testing this hypothesis would be greatly facilitated by cloning the genes encoding the binding sites, and these efforts are already under way.[62]

An extremely important issue for management of insecticide resistance in the field is the initial frequency of a resistant allele prior to the onset of strong pesticide selection. Conventional thinking regards this initial frequency to be set by a balance between mutation and selection, with mutation creating the resistant allele and selection tending to eliminate it from the population (it is implicitly assumed that the resistant allele is deleterious in the absence of the pesticide). Many mathematical models of the selective response to pesticides assume that this initial frequency is within the range 10^{-8} to 10^{-5}. Attempts to measure directly the initial frequency are rarely made, as this would clearly require sampling an enormous number of individuals from the field before the widespread use of a new insecticide.

Yet a recent attempt[63] to measure the initial frequency of the resistant allele at *BtR-4* was successful. Since the initial allele frequency was expected to be low, efficient estimation required the ability to detect heterozygotes, which carry the majority of resistant alleles present in the species' gene pool. Furthermore, since the *BtR-4* resistant allele is recessive, heterozygotes could not be directly detected, but instead had to be indirectly ascertained by testing their progeny. More than 2000 field-collected males were individually mated to YHD2 females, and progeny of more than 1000 of these were tested on artificial diet with a Cry1Ac concentration high enough to stunt the growth of all but homozygous resistant larvae. Most males produced only susceptible offspring, but four males produced both susceptible and resistant offspring. Resistant offspring of three of these males were intercrossed and their progeny were also resistant. The simplest explanation of these results is that each of the four males was heterozygous, contributing the susceptible allele at the *BtR-4* locus to some progeny and the resistant allele to the others (the YHD2 mother always contributed a resistant allele).[63] This yields an estimate of the initial resistant allele frequency of about 2×10^{-3}.

The magnitude of the resistance conferred by *BtR-4* and the unexpectedly high initial frequency of the resistance allele strongly suggest that field populations of *H. virescens* have the potential to rapidly attain resistance to transgenic cotton expressing Cry1Ac. Fortunately, from the very first commercial use of transgenic cotton in the US, a resistance management strategy has been mandated, which incorporates on-farm refugia for *H. virescens* that are not selected with Bt. The intent of this strategy is to produce enough susceptible individuals to interbreed with any resistant survivors of the transgenic cotton, and thereby dilute the frequency of the resistance allele.[64] Cloning *BtR-4* should be a high priority, to enable an understanding of the mechanism of resistance, to facilitate more efficient ways of measuring allele frequencies in field populations, and to verify whether the resistance management strategy based on untreated refuges is having the desired effect of keeping the resistant allele frequency low.

6 RESISTANCE MECHANISMS AND PROSPECTS FOR LINKAGE MAPPING IN *HELICOVERPA ARMIGERA*

Not to be outdone, *H. armigera* has developed resistance to DDT, organophosphates and pyrethroids and is also likely to become resistant to Bt. Resistance is widespread in China, India, Australia and Thailand and has been documented from many other countries.[65–72] OP and carbamate resistance is due to decreased penetration, increased detoxification and/or insensitive AChE.[73–75] Pyrethroid resistance is caused by decreased penetration, increased hydrolysis, increased metabolism by monooxygenases and/or nerve insensitivity.[39,76–81] Thus *H. armigera* possesses a wealth of resistance mechanisms that provide additional opportunities for linkage mapping.

As a first step in pursuing this approach, we are currently constructing a linkage map for *H. armigera.* Use of 'anchor' loci—the same isozymes and RFLPs already mapped in *H. virescens*—will facilitate map construction, and simultaneously enable comparisons between the linkage groups of the two species. This will enable testing of our working hypothesis that the two species will show a high degree of conservation of the identity of linkage groups and gene order within them, because of the relatively recent evolutionary divergence of the two genera. The 'anchor' loci should eventually prove useful in other interspecific comparisons among insects as well.[82]

When Bt-resistant strains of *H. armigera* become available for study, it will be possible to determine whether the genetic basis of resistance is similar to the *BtR-4* locus in *H. virescens.* Linkage of Bt resistance in *H. armigera* to the equivalent of *H. virescens* Linkage Group 9 would suggest that the two species have homologous resistance mechanisms. If so, then positional cloning of *BtR-4* in *H. virescens* could greatly aid the understanding of Bt resistance in *H. armigera.*

7 CONCLUSIONS

Improvement of genetic technology to enable rapid scoring of hundreds of genetic polymorphisms from a sample containing a microgram or less of DNA has now made construction of genetic linkage maps feasible in any species with sexual reproduction. There is now no need to confine genetic studies of resistance mechanisms to well-studied model systems such as *Drosophila*

and houseflies. The linkage mapping approach can sort out multiple resistance mechanisms in a single strain, differentiate between major and minor resistance loci, and provide useful information even when the mechanism of resistance is unknown. It can enable testing of hypotheses on the role of known genes in resistance, or provide the first step in positional cloning when the identity and products of the resistance genes are unknown. Although the results from *Heliothis* so far have shed light mostly on resistance mechanisms already developed and now widespread, the real challenge to the linkage mapping approach will be in anticipating resistance, i.e. in detecting and exploring resistance mechanisms while there is still time to implement resistance management strategies to delay and perhaps even prevent the spread of resistance in field populations.

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